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Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

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Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

R C van Dijk

DEN HAAG, DEN
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NMI NATURWISSENSCHAFTLICHES UND MEDIZINISCHES INTITUT AN DER UNIVERSITÄT TUBINGEN
72770 Reutlingen
GERMANY

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Method for determining analyte molecules in a sample

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Witte, Weller & Partner
Patentanwälte

Rotebühlstraße 121 . D-70178 Stuttgart

Applicant:

**NMI Naturwissenschaftliches und
Medizinisches Institut an der
Universität Tübingen
Markwiesenstraße 55**

D- 72770 Reitlingen

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3605P118 HO**

Method for determining analyte molecules in a sample

The invention relates to the field of chemical analysis, relating particularly to the field of diagnostic analysis and most particularly to protein detection and analysis, proteomics and clinical screening procedures.

Especially, the application relates to a method for determining, preferably quantifying analyte molecules in a sample and to a kit that comprises reagents suitable in performing the method.

One example of a generic detection method is a so-called Sandwich-Immune-Assay that might be performed on a microarray. Analytical procedures using microarrays are known in the art. Capture molecules or antibodies with a known affinity to an analyte are immobilised and the amount of analyte bound to the capture molecules is measured. The binding of the capture antibody is facilitated by specific modifications on the surface of the support. After binding of the capture antibody an incubation with individual samples is performed followed by reaction with a labelled probe, that can be e.g. a second sample-directed antibody suitable for completing the antibody-sample 'sandwich'.

The detection antibody is labelled with a radio- or fluorescence label or, in an enzyme-linked immunosorbant assay ("ELISA"), is coupled to an enzyme in order to catalyze a chemical reaction generating a detectable precipitate as product In each case, the primary data are measured and the amount of captured analyte can be calculated using defined concentration of the analyte in an unknown sample.

Microarray based procedures using this principle have been described in the prior art. One method relies on the assumption that the amount of captured analyte does not change the concentration of the analyte in the sample significantly and therefore the mass action law can be used to calculate the concentration in the unknown sample (ambient analyte assay systems). However, in many application this assumption fails to lead to reliable results.

Competitive Assays for the quantification of an analyte can also be used to determine unknown concentrations in a sample. Commonly used assays are the competitive radioactive immunosorbant assay (RIA) procedures and the competitive enzyme linked immunosorbant assay (ELISA). In both cases a labelled analyte to be measured is included in the reaction mixture. During sample incubation a competitive reaction between labelled standard and sample protein creates a signal describing which is used to determine the analyte concentration via a series of standards.

This prior art is accompanied by several severe disadvantages, namely:

- to calibrate the assay several standards have to be included to allow a reliable quality control; multiple standards are needed;

- Quality of the obtained data is highly dependent on the quality of the spot and methods to correct or to minimise spot to spot variation have to be introduced to obtain reliable results;

- the described microarray based systems have to use an optimal working concentration that is determined by the binding constants (capture molecule and analyte) and the amount of the capture molecules used. Therefore, the concentration of the analyte in the assay has to be adjusted to an optimal value to allow the method to work reliably. This points to a principle disadvantage of the described assay systems in general. When applying the method for a simultaneous detection of multiple analytes in one assay, it is difficult to adjust the concentration of each analyte to be quantified to its optimal value. This presents a major problem since the concentration of different analytes to be quantified may vary by several orders of magnitude. The problem is a major obstacle when using the 'ambient analyte assay systems' as described by Ekins, because measurements performed at non optimal conditions suffer from a high intrinsic experimental error.

U.S. 5,834,319 discloses a method for determining the concentration of an analyte by binding it to a surface in non-saturating quantities. A back-titration is then made with a labelled analogue of the analyte , i.e. a first developing reagent, followed by reaction with a second developing reagent, which is also labelled. Not only is a labelled analyte analogue required in this m thod, but this back titration requires that the labelled analogue be added after the ambient assay to saturate binding sites which were not occupied by the analyte in the sample, thereby extending the

procedure and entailing additional washing steps in which bound analyte dissociates from the captur molecules.

WO 00/63701 discloses microarrays of polypeptides on a solid support for multiplexed detection and quantitation of ligands. The ligands are labelled directly and detected without a detection reagent being used. The method is therefore inflexible in terms of responsiveness and range of analyte concentrations over which it may be usefully employed.

In view of the above, it is an object of the present invention to avoid and/or circumvent the drawbacks of the prior art and to provide a reliable, easy to perform method for determining the amount or concentration of an analyte in a sample.

This object is achieved with a method as detailed in claim 1, a kit as in claim 11 and the use of arrays and beads as in claims 15 or 17. Preferred embodiments of the invention are subject of the subclaims.

The invention can be performed in solution, suspension (beads, virtual array) or in an array format. An important feature of the invention is that the competitor/analogue molecules are distinct from the analyte molecules, i.e. can be distinguished therefrom. To this end the analyte and/or competitor/analogue molecules can be labelled or conjugated e.g. to an enzyme. In case the analyte molecules are already labelled, unlabelled competitors can be used, a detection reagent being provided that allows determination of the sum of analyte and competitor/analogue molecules bound to the capture molecules.

Another option is to use at least two differently labelled antibodies that bind the analyte and the competitor, respectively.

The binding constant between analyte and capture molecules can be equal to or different from the binding constant between comp titor and capture molecules. The relationship between the concen-

trations/amounts of analyte and competitor molecules can either be calculated based upon the law of mass action or taken from a calibration curve.

The inventive method has several advantages:

The need for standards is reduced. It is sufficient to know the exact amount of competing, preferably labelled analyte analogue or competitor present in the assay to get valid information about the concentration to be determined. It is not necessary to know the concentration of the capture molecules.

The invention allows to analyse a large number of analytes simultaneously, even when their concentrations are different. The assay can be performed under saturating conditions were conventional systems don't work any more, since the amount of added standard (competitor), which is for generating the primary signal, can be adjusted individually. The method as such provides internal standards for quantification, leading to reliable and robust assay procedures. Inter-assay and intra-assay variations are efficiently eliminated by internal standardisation. Assay is not dependent on the affinity constants of the capture molecules. Characterised capture and detection reagents (eg. ELISA reagents) can be employed with minimal operating expense .

A new combination of competitive and non-competitive assays in one reaction is provided. The method helps to circumvent the major disadvantages of prior art microarray based detection systems. This new combination allows the parallel detection of multiple parameters and therefore allows to determine the concentration of a high number of analytes at the same time.

Conventional assay systems using protein microarrays depend on the optimal amount of capture molecule immobilised and on a fitting concentration of the analyte to b quantified. The 'ambient analyte assay system' as described in US 5,834,319 is working accurately only when a small (< 5%) proportion of the analyte gets bound to the

capture molecule. This is a major drawback wh n planning to quantify different analytes that are present in differing concentrations (differences of several orders of magnitude) in one sample using only one reaction.

The invention uses in a preferred embodiment the addition of an labelled analogue of the analyte to be determined. A known amount of the labelled analogue of the analyte is added to the sample and on the immobilised capture molecules the labelled analogue and the unlabelled analyte get captured. The ratio of the two substances captured on a micro-spot presents a value that can be used to calculate the unknown amount of analyte. To determine the ratio, the competing labelled analyte analogue is detected by its introduced label. The analyte or the sum of analyte and analyte analogue present in the sample is in a preferred embodiment detected by a specific detection reagent like a secondary antibody.

This detection reagent can be specific for both the analyte to be quantified and the labelled analogue of the analyte. The obtained measurements provide information on (i) the amount of competitor captured and (ii) on the sum of competitor and analyte captured. These data are also sufficient to calculate the amount of unknown analyte.

This detection reagent can be specific for the analyte to be quantified and therefore is not able to recognise the labelled analogue of the analyte. An assay of this type corresponds to a competitive system where competitor can be quantified and a simultaneous detection of analyte that is archived by a different system. Both reactions occur at the same time in the same assay and therefore can be used to quantify the analyte by two different methods at the same time.

Both methods are working independently of the actual concentration of the analyte. The assays can be performed wh n only a fraction of the analyte is captured by the immobilised capturing reagent sample (ambient analyte assay system, no significant change of the

concentration in the liquid phase) or when a significant proportion of analyte is removed from solution by the capture molecules. The method can be performed under conditions where the capture molecules are saturated by bound analyte (conventional systems don't work), since the ratio of the competing labelled analyte analogue and the analyte is used for the concentration determination.

Assay variations and spot irregularities do not influence the determined ratios significantly. The need for controls is greatly reduced.

Applications of the new method are:

Screening: Diagnostic screening procedures (concentration determination of immunoglobulins, interleukins, etc.), screening systems for the pharmaceutical industry.

Profiling of biomolecules: Quantification of high abundant and low abundant proteins or peptides; Quantification of high affinity and low affinity binding proteins or peptides, sugars, nucleic acids and the like.

Example: Quantification of IgG3

a) Reagents

1. Cy3 mAb Labelling Kit (PA33001) from Amersham-Pharmacia.
2. Filter spin units "Microcon 10" from Amicon.
3. Tween-20, TRIS-base, Thimerosal from Sigma Chemical Co. (St. Louis, MO, USA); bovine serum albumin Fraction V from PAA Laboratories; PBS from Eurobio; low fat dried milk from Heirle; sodium carbonate decahydrate from Fluka.
4. Aldehyde activated glass slides (silylated slid s CSS) from Telechem.

5. Rat anti mouse IgG3 monoclonal capture antibodies (Clone R2-38) and rat anti mouse IgG3 monoclonal Biotin-conjugated developing antibodies (Clone R40-82), Mouse IgG3 standard (Clone A112-3) from Pharmingen.
6. Cy5-conjugated Streptavidin from Amersham Pharmacia.

b) Creation of microarrays

Rat anti mouse IgG3 monoclonal capture antibodies (conc. 500µg/ml in PBS) were arrayed using a GMS 417 micro arrayer, three dots (equivalent to 150 pl) were deposited to give one spot. Arrays were created on aldehyde activated slides (CSS slides, Telechem). After printing the slides were incubated for 12 h in a wet chamber and stored dry in the dark.

c) Labelling and purification of proteins with fluorescent dyes

1. 100 µg IgG3 standard at a concentration of 1 mg/ml was used for the labelling reaction. Coupling was performed as described by the manufacturer.
2. To terminate the reaction 1M TRIS-HCl pH 8.0 was added to a final concentration of 100 mM and incubated in the dark for 30 min.
3. The labelling mix was transferred to a "Microcon 10" concentration unit, adjusted to 400 µl with PBS and centrifuged for 25 min at 12000 g. PBS was added to the protein solution to give a volume of 400 µl and the concentration procedure was repeated twice to wash away free label.
4. The purified Cy3-labeled IgG3 was brought to a volume of 200 µl by adding PBS containing 0.05% Thimerosal.

d) Combined competitive/non-competitive Assay for the quantification of IgG3

1. Slides were blocked in PBS containing 1.5% BSA and 5% low fat dried milk powder, 0.1% Tween-20 for 1h and washed briefly in PBS containing 0.1% Tween-20.

2. Serum samples were diluted (1:40, 1:60, 1:70, 1:80, 1:90, 1:100, 1:160) in 25 µl PBS containing 1.5% BSA, 2.5% low fat dried milk and Cy3-labeled IgG3 was added as competitor (1:1000). The arrays were incubated with the sample containing analyte and competitor for 40 min at RT. The relative concentration ratios between competitor and analyte were thus: 1:25, 1:16,67, 1:14,29, 1:12,5, 1:11,1, 1:10, and 1:6,25, respectively.

3. The arrays were washed five times in PBS, 0.1% Tween-20 for 1 min at RT.

4. Biotin-conjugated developing antibody was diluted in PBS containing 1.5% BSA and 2.5% low fat dried milk powder, 0.1% Tween-20 1:800 (0.625 µg) and the array incubated therewith for 40 min at RT and washed as described under 3.

5. Streptavidin-Cy 5 was diluted 1/200 in PBS containing 1.5% BSA and 2.5% low fat dried milk powder, 0.1% Tween-20. The assays were incubated therewith for 20 min at RT and washed as described under 3.

6. The slides were rinsed once with water, once with distilled water and dried in a stream of nitrogen. Pictures of the fluorescent signals of Cy3 and Cy5 dyes were taken using a GMS418 array scanner. Quantification of the obtained fluorescence signals was done with the ImaGene 4.0 software. The ratio of the Cy5 and the Cy3 signals was calculated and plotted against the relative concentration (1:160 dilution was set to 1) of the analyte.

Fig.1 shows a sketch of this plot.

Example 2: Quantification of recombinant IgG

Essentially the same experimental procedures as d scribed above were applied to a diff rent type of sample (recombinant IgG).

In contrast to the first exampl the concentration of analyte in this experiment was varied from 3, 3, 27, 81, 243, 729. Competitor was diluted 1:1000.

Fig.2 shows a sketch of the obtained data.

Both Fig. 1 and 2 show that once a calibration curve or numerical relationship between the determined ratio and the relative analyte concentration/amount has been obtained, unknown concentrations/-amounts of analyte molecules can be determined from the ratio and the known concentration/amount of competitor/analogue molecules. The concentration/amount can either be in spread narrow range or a wide range as in Fig. 2.

Claims

1. A method for determining the concentration/amount of analyte molecules in a sample, comprising the steps of:

a) adding to the sample a known concentration/amount of competitor or analogue molecules of the analyte, said competitor/analogue molecules being distinct from said analyte molecules,

b) reacting/contacting the sample of step a) with capture molecules for the analyte and competitor molecules,

c) determining the ratio of analyte and competitor molecules that are bound to the capture molecules,

d) determining the concentration/amount of the analyte molecules from the ratio of step c) and the concentration/amount of competitor molecules in the sample of step a).

2. The method of claim 1 or 2, wherein in step c) the amount of competitor molecules bound to the capture molecules is determined using a detection reagent, preferably a labelled or (enzyme) conjugated ligand.

3. The method of claim 1 or 2, wherein in step c) the amount of analyte molecules bound to the capture molecules is determined using a detection reagent, preferably a labelled or (enzyme) conjugated ligand.

4. The method of any of claims 1-3, wherein in step c) the total amount of
analyte and competitor molecules bound to the capture molecules is determined using a detection reagent, preferably a labelled or (enzyme) conjugated ligand.

5. The method of any of claims 1-4, wherein in step c) the amount of competitor molecules bound to the capture molecules is determined distinct but parallel to the determination of analyte molecules bound to the capture molecules.

6. The method of any of claims 1-5, wherein in step b) analyte and competitor molecules not reacted with/bound to the capture molecules are removed, preferably by washing steps.

7. The method of any of claims 1-6, wherein the method is performed on an array of immobilised capture molecules.

8. The method of any of claims 1-6, wherein the method is performed in a suspension of beads having attached thereto capture molecules.

9. The method of claim 1 or any of claims 3-8, wherein the competitor molecules are labelled, preferably with a fluorescent dye.

10. The method of any of claims 1-9, wherein the analyte molecules are labelled, preferably with a fluorescent dye.

11. Kit for performing the method of any of claims 1-10, comprising capture molecules and preferably detection reagents for analyte and/or for competitor molecules.

12. The kit of claim 11, further comprising competitor molecules.

13. The Kit of claim 11 or 12, wherein the capture molecules are arranged in an array, preferably a microarray.

14. The kit of claim 11 or 12, wherein the capture molecules are attached to beads.

15. Use of an array, preferably a microarray of capture molecules for determining the concentration/amount of at least one species of analyte molecules, in a method as in any of claims 1 -10.

16. Use according to claim 15, wherein said array comprises different species of capture molecules for determining different species of analyte molecules.

17. Use of a suspension/virtual array of beads having attached thereto capture molecules for determining the concentration/amount of at least one species of analyte molecules, in a method as in any of claims 1 -10.

18. Use according to claim 17, wherein said beads have attached thereto different species of capture molecules for determining different species of analyte molecules.

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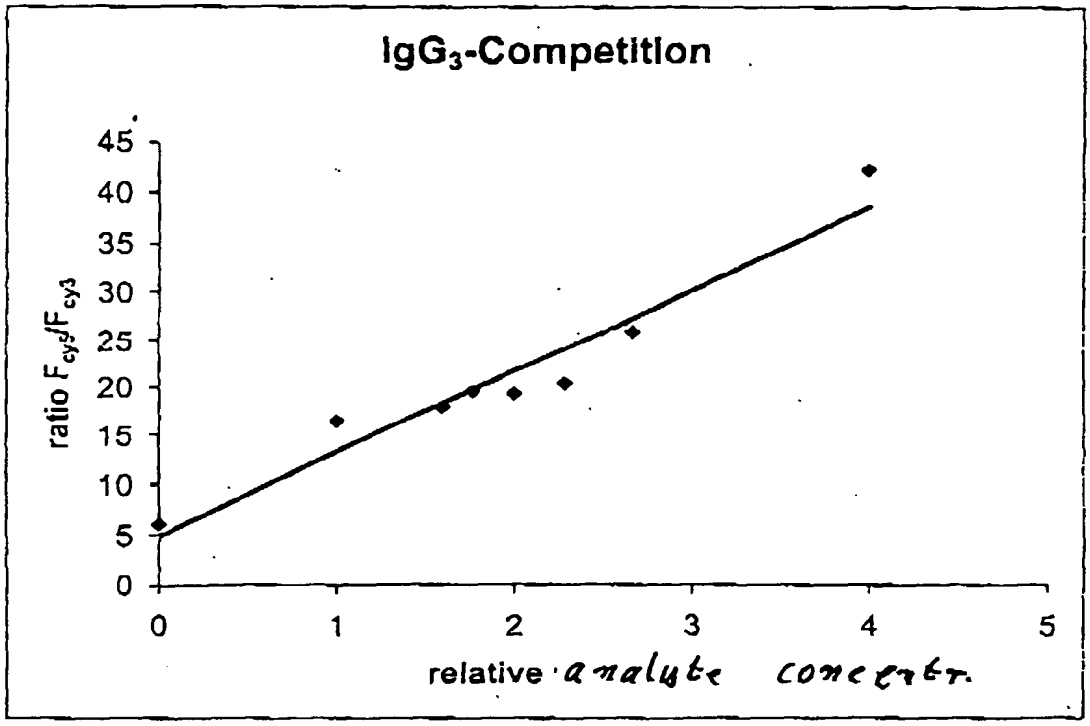
IgG3-Competition
ratio Fcy5/Fcy3
45
40
35
30
25
20
15
10
5
0
0
1
2
3
4
5
relative analyte concentr.

Fig. 1

2/2

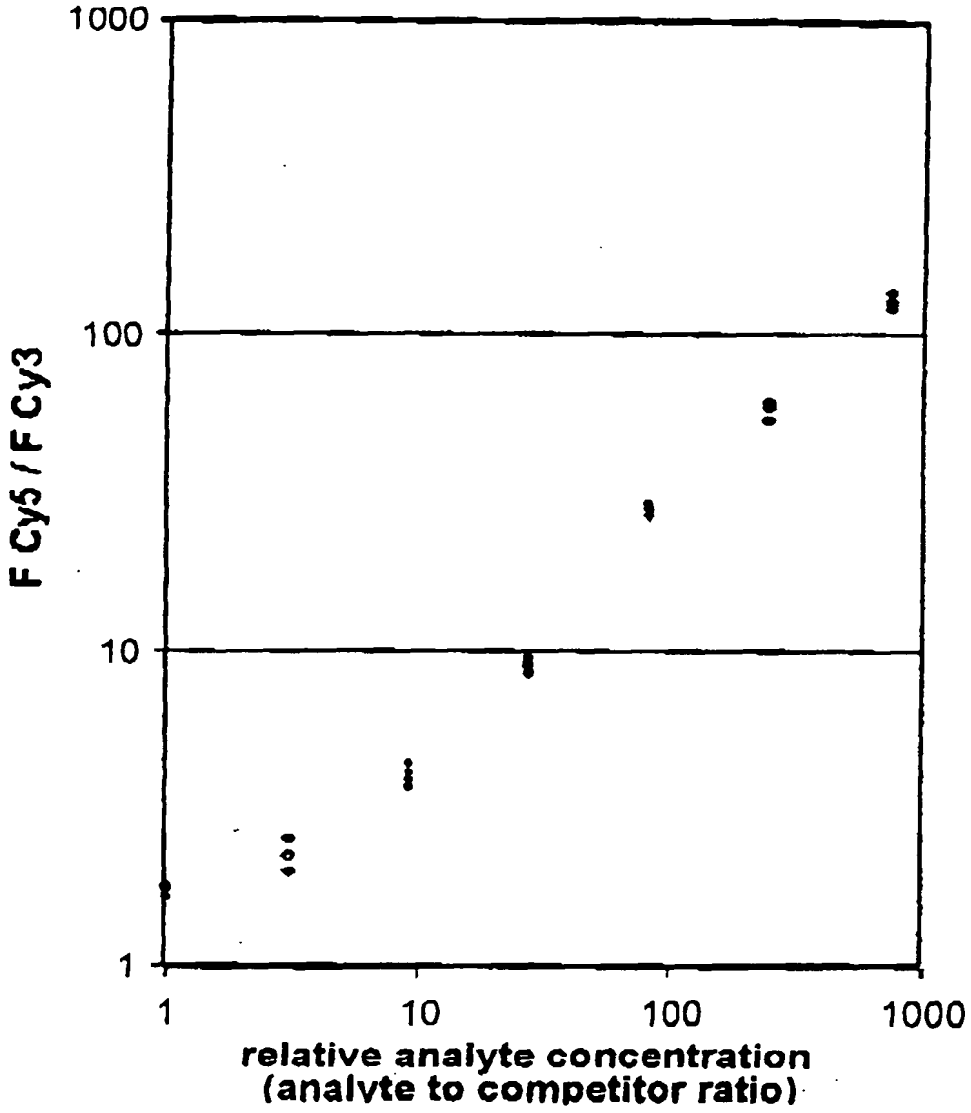
1000
100
10
1
F Cy5 / F Cy3
1
10
100
1000
relative analyte concentration
(analyte to competitor ratio)

Fig. 2